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Clinical Study of the Relationship Between Cytological Behavior and Postoperative Prognosis in Colorectal Cancer Cases With Special Reference to Nuclear DNA Content and Nucleolar Organizer Regions

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Background: We studied the usefulness of nuclear DNA patterns and argyrophylic nucleolar organizer regions (AgNORs) for evaluating the malignant potential of colorectal cancers, which is increasingly being regarded as important in predicting patients' prognosis and for their appropriate postoperative management.
Methods: We measured these two factors in curatively resected specimens of 91 colorectal cancer cases, which were followed up for 1,549 ± 788 days postoperatively. Ploidy pattern was either diploid or aneuploid, and AgNORs score was either low (LS) or high (HS). Thus, we classified our cases into Group I (diploid, LS), Group II (aneuploid, LS), Group III (diploid, HS), and Group IV (aneuploid, HS). Postoperative survival curves in the cases belonging to these groups were analyzed.
Results: Survival rates in Groups I and II were significantly higher than those in Group IV. Correlation between subgroups and clinicopathological factors such as average age, histologic type, depth of invasion, and histologic stage were observed. Incidence of lymph node metastasis at the time of operation and that of postoperative recurrence were higher in group IV than that in groups I and II.
Conclusions: Measurement of DNA ploidy patterns and AgNORs score were found to be useful in evaluating malignant potential of colorectal cancers. J. Surg. Oncol. 64:36–41

KEY WORDS: colorectal cancer; DNA ploidy pattern; AgNORs score

INTRODUCTION

The prognosis of colorectal cancer cases has been usually classified and evaluated according to the staging defined by clinicopathological findings. However, this staging is mainly based on the degree of cancer advancement and is not always associated with the biological malignant potential of cancer, which is necessary to predict the biological aggressiveness of a tumor and useful for appropriately managing postoperative patients. Nuclear DNA contents have been considered as a useful index of estimating biological malignancy [1–3], because the proliferating potential of the cancer cell is strongly associated with nuclear DNA contents [4]. However, nucleolar organizer regions (NORs) are DNA loops that code ribosomal RNA (rRNA). They converge in the

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nucleolus in the resting period and exist at the second stricture on the 13, 14, 15, 21, and 22 chromosomes in the fission period. These NORs are closely related to production of ribosome and protein through transcription of rRNA and reflect the activity of the nucleolus and the whole cell. Argyrophilic NORs (AgNORs) stain [5] is the way to depict specifically nonhistone proteins binding to NORs and is associated with fission and proliferating activity of the cell.

We measured nuclear DNA contents and AgNORs using paraffin-embedded specimens of colorectal cancer to investigate the relationship between the degree of biological malignancy and prognosis of the patients.

MATERIALS AND METHODS

From July 1984 through June 1988, 91 consecutive primary colorectal cancer patients underwent curative operation and remained on follow-up for 1,549 ± 788 days postoperatively. Postoperative recurrence was observed in 21 of the 91 patients. DNA ploidy patterns and AgNORs were examined using the resected specimens obtained from these 91 patients. Their clinicopathological characteristics were analyzed according to the Japanese general rules on colorectal cancer [6]. These characteristics consisted of sex, age, tumor location, tumor diameter, histologic type, depth of invasion, lymph node metastasis, venous invasion, lymphatic invasion, liver metastasis, peritoneal dissemination, histologic stage, and incidence of recurrence.

DNA ploidy patterns were measured according to the method of Schutte et al. [7] and Vindelov et al. [8]. Nuclear DNA contents were measured in 10,000 cancer cells using Facstar (Becton-Dickinson Immunocytometry Systems, San Jose, CA), after nuclear stain. DNA histograms were classified into two groups: diploid and aneuploid. In the former, the G0/G1 peak was single and DNA index (DI) was 1.00. In the latter, there were one or more peaks other than the G0/G1. Specimens having more than eight coefficient variations were excluded. Silver stain for AgNORs was performed by Ploton's modified one-step silver method [9]. AgNOR scores were calculated by Howat's method [10] as follows. The number of silver dots were counted on 100 cancer cells at 1,000 power magnification. The total number of dots was divided by the total number of cell nuclei in the same visible field. Count was taken in the deepest region of the tumors. Only the specimens in which one clear AgNOR was recognizable in one small lymphocyte at the interstitial space, were used for the count.

According to both factors, the total cases were subdivided into the following four groups: Group I (diploid, low score), Group II (aneuploid, low score), Group III (diploid, high score), and Group IV (aneuploid, high score). The values were indicated with mean ± standard deviation. Differentiation between two groups were analyzed statistically, using generalized Wilcoxon tests when the measurement values were continuous numbers or using Chi-square tests when they were category numbers. Survival rates were indicated with accumulated rates calculated by Kaplan-Meier method and tested by log rank test. Differences were considered significant when P was <0.05.

TABLE I. Characteristics of 91 Colorectal Cancer Patients and Their Clinicopathological Findings

Characteristics		Cases (91)
Sex	male	54
	female	37
Age (year)	30–39	4
	40–49	10
	50–59	21
	60–69	26
	70–79	26
	80–	4
Average (mean ± SD)		62.2 ± 11.5
Histologic type	well[a]	42
	mod[b]	38
	por[c]	7
	sig[d]	1
	others	3
Histologic stage	I	24
	II	36
	III	11
	IV	20
	V	0

[a]Well-differentiated adenocarcinoma.
[b]Moderately differentiated adenocarcinoma.
[c]Poorly differentiated adenocarcinoma.
[d]Signet ring cell carcinoma.

RESULTS

The background characteristics of the 91 colorectal patients (54 males and 37 females) evaluated in this study are shown in Table I.

Primary Tumor

Fifty-one cases (56.0%) were in the diploid group and 40 cases (44.0%) in the aneuploid group. The average DNA index (DI) was 1.23 ± 0.32 in all cases but 1.51 ± 0.30 in the aneuploid group. The AgNORs scores of the 91 cases were from 2.68 to 4.78, the average being 3.75 ± 0.39. They were divided into the low score (LS) group, in which the scores were <3.75 and the high score (HS) group, in which the scores were 3.75 or more. A significant correlation was observed between DNA ploidy patterns and AgNOR scores ($P < 0.05$, Table II). Twenty-three cases (57.5% of the aneuploid cases) were in the HS group and 23 cases (56.1% of the HS cases) were in the aneuploid group.

Taking the above facts into consideration, we subdivided the total cases into the following four groups: Group I (diploid, LS). Group II (aneuploid, LS), Group

TABLE II. Subclassification of 91 Colorectal Cancer Patients According to DNA Ploidy Pattern and AgNORs Score

	Low score (AgNORs score < 3.75)		High score (AgNORs score ⩾ 3.75)		Total
Diploid	Group I	33	Group III	17	50
Aneuploid	Group II	18	Group IV	23	41
Total		51		41	91

$P < 0.05$ (x^2 test).

III (diploid, HS), and Group IV (aneuploid, HS). The clinical findings and prognosis in these groups were analyzed and compared with each other (Table III). Ninety-one cases were divided into 33 Group I cases, 18 Group II cases, 17 Group III cases, and 23 Group IV cases. The age of 66.2 ± 9.1 in Group I was significantly older than that of 56.9 ± 12.2 in Group III ($P < 0.05$). In terms of depth of invasion, Group I included more m (mucosa), sm (submucosa), and pm (proper muscle) than Groups II and IV ($P < 0.01$, $P < 0.05$, respectively). Group IV included more positive cases of lymph node metastases than Groups I and II ($P < 0.01$, $P < 0.05$, respectively). Histologically, Group I included more stage I tumors than Groups III and IV ($P < 0.05$, $P < 0.01$, respectively). Postoperative recurrence was seen in 21 of the 91 cases. There were 4/33 (12.1%) Group I cases, 3/18 (16.7%) Group II cases, 4/17 (23.5%) Group III cases, and 11/23 (47.8%) Group IV cases. Thus the recurrence rates increased in the numerical order of Groups I, II, III, and IV. There were significant differences ($P < 0.05$) between Groups I and IV, and Groups II and IV, respectively. There was no significant difference in the recurrence patterns among those groups in the colon cancer cases, but Group IV included more recurrent cases than Group I in the rectal cancer cases (Table IV). The recurrence occurred at a single site, such as liver or local area in Groups I and II, whereas hematogenous recurrences in multiple organs were frequently observed in Group IV. Eight of nine cases were hematogenous recurrence in the colon cancer cases, whereas 8 of 12 cases were local recurrence in the rectal cancer cases.

Group I had the best prognosis, Groups II and III were next and Group IV had the poorest prognosis. Groups I and II fared significantly better than Group IV ($P < 0.05$) and Group I tended to do better than Group III ($P = 0.062$, Fig. 1).

Comparison Between Primary Tumor and Metastatic Lymph Node Lesion

We investigated nuclear DNA contents and AgNORs in the 28 cases with positive lymph node metastasis, out of the 91 curatively resected cases (Table V). There were 13 (46.4%) metastasis-positive cases in Group IV and 19 (67.9%) cases in Group III+IV. Groups III and IV tended to include relatively more stage IV cases. Recurrence rates were 33.3% in Group I, 33.3% in Group II, 66.6% in Group III, and 61.5% in Group IV.

Discrepancy in DNA index of the primary tumor and the metastatic lesions was seen in 3/19 (15.8%) cases and in the AgNORs score it was seen in 2/19 (10.5%) cases (Fig. 2). Discrepancy in both factors was seen in only one case. Discrepancy in AgNORs score was not seen in any of the 11 patients who survived without recurrence. Out of eight patients who succumbed to recurrence or are surviving with recurrence, two cases showed discrepancy in DNA ploidy and two cases showed it in AgNORs. One case, in which discrepancy was seen in both AgNORs and DNA ploidy, succumbed to local and hepatic recurrence, 2 years 9 months after operation. One case, in which discrepancy was seen in DNA ploidy but not in AgNORs, is surviving with hepatic recurrence. One case, in which discrepancy was seen in AgNORs but not in DNA ploidy, also succumbed to hepatic recurrence, 3 years after operation. In three cases, which succumbed to recurrence in <3 years after operation, metastatic lymph nodes showed an aneuploid pattern and high AgNORs score. In five cases that survived for >3 years after operation, metastatic lymph nodes were diploid in four cases and aneuploid in one case.

DISCUSSION

Yamaguchi et al. [11] investigated AgNORs score (AS) in colorectal cancer specimens obtained under colonoscopy. They classified their specimens into the low score (LS) group and the high score (HS) group and reported that there was a significant difference between both groups in DNA ploidy patterns. In our investigation, using materials obtained at operation, there was a significant correlation between AS and ploidy patterns. Group I (diploid, LS) had the best and Group IV (aneuploid, HS) had the poorest prognosis. Recurrence rates were 12.1% in group I, 16.7% in Group II (aneuploid, LS), 23.5% in Group III (diploid, HS), and 47.5% in Group IV. Thus in the low AS cases, the recurrence rate was low irrespective of DNA ploidy patterns, whereas in the high AS cases, the recurrence rate was fairly high. In other clinicopathological findings, there were also a number of significant correlations among those groups, which indicate progressiveness of the cases in Group IV, although there were a rather small number of patients in each group. The above data demonstrate that the measurement of these two factors might provide an accurate, quantitative predictor of the biological behavior upon which to base future patient management.

We have found no previous study comparing DNA ploidy patterns and AS of the primary tumor and metastatic lymph node lesions in colorectal cancers. We investigated the lymph node lesions in 19 of the 28 cases that underwent curative colectomy with positive lymph

TABLE III. Clinicopathological Findings of Colarectal Cancer Patients Who Underwent Curative Operation Among Subclasses According to the Combination of DNA Ploidy Pattern and AgNORs Score*

Characteristics		Group I	Group II	Group III	Group IV	Kruskal-Wallis test
Number of cases		33	18	17	23	
Male:female		22:11	10:8	10:7	12:11	
Age (mean ± SD)		66.2 ± 4.1	63.2 ± 13.9	56.9 ± 12.2	54.8 ± 10.4	I–III:$P < 0.05$
Location	colon (rectum)	19 (14)	11 (7)	7 (10)	16 (7)	
Depth of invasion[a]	m,sm	8	1	0	0	
	pm	12	2	3	2	I–II:$P < 0.01$
	ss(a1)	7	11	13	18	I–IV:$P < 0.05$
	s(a2)	4	2	1	2	
	is(a3)	2	2	0	1	
Lymp node metastasis	n(−)	27	15	11	10	I–IV:$P < 0.01$
	n(+)	6	3	6	13	II–IV:$P < 0.05$
Histologic stage	I	19	3	1	1	
	II	6	11	10	9	I–III:$P < 0.05$
	III	5	3	1	2	I–IV:$P < 0.01$
	IV	3	1	5	11	
Recurrence	(−)	29	15	13	12	I–IV, II–IV:$P < 0.05$
	(+)	4	3	4	11	

*There was no significant difference in tumor diameter, histologic type, venous invasion lymphatic invasion, liver metastasis, and peritoneal dissemination.

[a]m:mucosa, sm:submucosa, pm:proper muscle, ss(a1):subserosa (subadventitia), s(a2):serosa (adventitia), si(a3):infiltration beyond serosa (adventitia).

TABLE IV. Relationship Between Type of Recurrence and Subclass According to Combination of DNA Ploidy Pattern and AgNORs Score

A. Colon cancer

Type of recurrence	Group I (19)	Group II (11)	Group III (7)	Group IV (16)
Liver	2	1	0	3
Local	0	0	0	0
Peritoneum	0	0	0	1
Hematogenous (multiorgan)	0	0	0	2
Local + hematogenous	0	0	0	0
Total	2(10.5%)	1(9.1%)	0(0%)	6(37.5%)

B. Rectal cancer

Type of recurrence	Group I (14)	Group II (7)	Group III (10)	Group IV (7)
Liver	0	0	1	1
Local	2	1	1	1
Peritoneum	0	0	0	0
Hematogenous (multiorgan)	0	0	0	2
Local + hematogenous	0	0	2	1
Total*	2(14.3%)	1(14.2%)	4(40%)	5(71.4%)

*I–IV : $P < 0.05$

node metastasis. In the remaining nine cases, metastatic lesions were too small in quantity or poor in quality to measure. Discrepancy in DNA ploidy patterns between

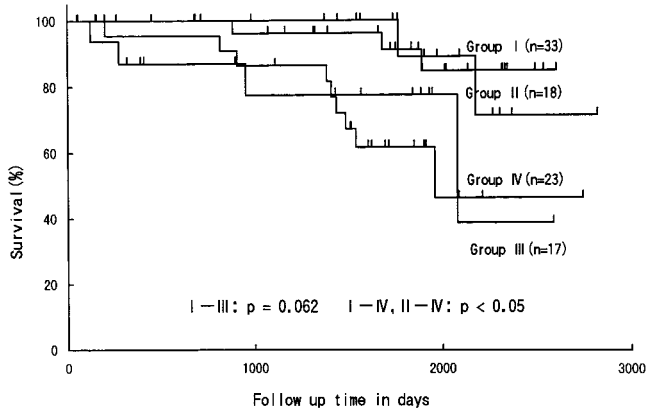

Fig. 1. Survival curves in four groups, classified according to the combination of DNA ploidy patterns and AgNOR scores in 91 patients with colorectal cancer who underwent curative resection

the primary tumor and the metastatic lesions was seen in 3 of the 19 cases. The two cases, who had aneuploid primary tumor and diploid metastatic lesions, survived for >5 years postoperatively, but the one case who had diploid primary tumor and aneuploid metastatic lesions succumbed to local and hepatic recurrence within 3 years after operation. Regarding the relationship between the primary tumor and lymph node metastases, Ushida et al. [12] reported that there was no significant difference in DI value among 21 colorectal cancer cases. On the contrary, Koha et al. [13] reported that 17% (3/18) of cases revealed discrepancies in DI value. Baretton et al. [14] investigated their 163 cases and found that 38% of the

TABLE V. Significance of Clinicopathological Findings on subclass With Positive Lymph Node Metastasis of Curatively Resected Colorectal Cancer

Characteristics		Group I	Group II	Group III	Group IV	Kruskal-Wallis test
Number of cases		6	3	6	13	
Male:female		2:4	0:3	4:2	6:7	
Age(mean ± SD)		63.4 ± 10.7	63.5 ± 15.5	48.7 ± 12.9	59.5 ± 10.2	n.s.
Histologic type[a]	well	3	1	1	5	
	mod	2	2	3	5	n.s.
	por	1	0	2	2	
	others	0	0	0	1	
Depth of invasion[b]	pm	1	2	1	2	
	ss(a1)	2	1	4	11	n.s.
	ss(a2)	3	0	1	0	
Histologic stage	III	5	3	1	2	
	IV	3	1	5	11	
Recurrence	(−)	4	2	2	5	n.s.
	(+)	2	1	4	8	

[a]well:well-differentiated adenocarcinoma, mod:moderately differentiated adenocarcinoma, por:poorly differentiated adenocarcinoma.
[b]pm:proper muscle, ss(a1):subserosa(subadventitia), s(a2):serosa(adventitia).
n.s. not significant.

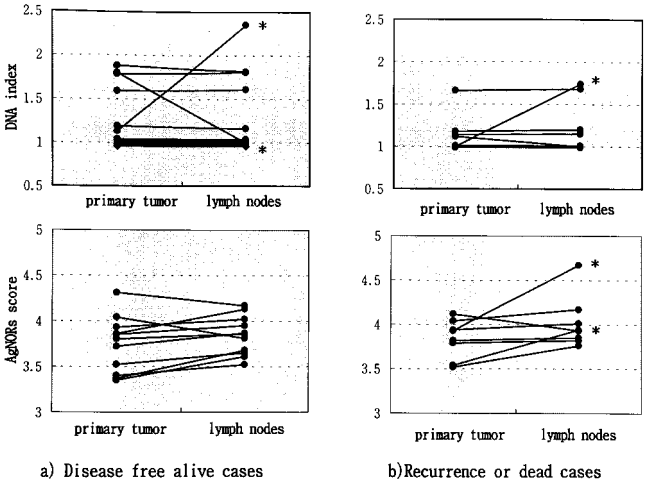

Fig. 2. Comparison of DNA index and AgNOR score between primary tumors and metastatic lymph node lesions in curatively resected cases. **(a)** disease-free, alive cases, **(b)** recurrent alive or dead cases. *Cases showing discrepancy between primary tumor and metastatic lymph nodes.

cases in which the primary tumor was aneuploid and 6% of the cases in which the primary tumor was diploid showed discrepancy in DI value. They also reported that the aneuploid cases showed relatively frequent recurrence and their prognosis was generally poor. That report coincides with our results. However, there have been few reports referring to AgNORs in metastatic lymph nodes. Moran et al. [15] reported that there was no difference between primary tumor and metastatic lesions. We found that the average value of AS in the primary tumor (3.78 ± 0.25) was significantly lower than that of metastatic lymph nodes (3.92 ± 0.26), and it is suggested that proliferative potential is increased in metastatic lymph nodes that are advancing portions of cancer. The two cases in which consecutive AS values at metastatic lesions increased from 3.54 to 3.94 and from 3.93 to 4.67, respectively, succumbed within 3 years after operation. All the positive metastatic cases, which succumbed within 3 years after operation in spite of curative resection, revealed aneuploid and high AS value (>3.92) in the metastatic lesions. Four out of the five cases that survived for >3 years postoperatively were diploid, and only one case was aneuploid in the metastatic lesions.

The above results suggest that the measurements of DNA ploidy patterns and AgNORs in metastatic lymph nodes are effective in evaluating the biological malignant potential of tumors and that the prognosis of the patients whose metastatic lymph nodes show aneuploid and HS is likely to be poor.

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